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THE EFFECT OF SOME *SOLANUM* STEROIDAL ALKALOIDS AND GLYCOALKALOIDS ON LARVAE OF THE RED FLOUR BEETLE, *TRIBOLIUM CASTANEUM*, AND THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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Key Word Index—*Solanum* spp.; Solanaceae; secondary plant compounds; spirosolane and spirostan derivatives; steroidal glycoalkaloids; solamargine; solasonine; tomatine; aglycones; solasodine; tomatidenol; tomatidine; diosgenin; cholesteryl tomatide; larval growth inhibition; red flour beetle, *Tribolium castaneum*; tobacco hornworm, *Manduca sexta*.

Abstract—Evaluation of the inhibitory effect of a series of secondary plant compounds including steroidal alkaloids and glycoalkaloids on larvae of the red flour beetle, *Tribolium castaneum*, was investigated. Larval growth was inhibited on artificial diets containing 1 μmol g^{-1} diet of the glycoalkaloids solamargine, solasonine and tomatine, whereas the corresponding aglycones solasodine and tomatidine, and also tomatidenol, were inactive. The inhibitory effect of solamargine and tomatine, but not of solasonine, was completely abolished by addition of 1 μmol g^{-1} diet cholesterol and/or sitosterol. Nonetheless, synthetic cholesteryl tomatide displayed significant activity at 2 μmol g^{-1} diet. Parallel studies with the tobacco hornworm, *Manduca sexta*, showed marked inhibitory activity of tomatine at a dietary concentration of 1 μmol g^{-1}, whereas the other compounds did not affect sterol metabolism or larval development. An appraisal of the factors influencing the mode of action of the active steroidal glycoalkaloids is attempted. © 1997 Elsevier Science Ltd.

INTRODUCTION

Early reports of allelopathic [1] and allelochemical [2] effects attributed to spirosolane glycoalkaloid-bearing *Solanum* species, led us to investigate the potential antifeedant/insecticidal activity of several solanaceous plants, and/or derivatives thereof, which have been obtained in connection with an extensive study on the development of convenient sources of raw steroids for the synthesis of steroidal drugs [3].

The presence of steroidal glycoalkaloids of the spirosolane series such as solamargine, solasonine, α- and β-solamarines and tomatine in over 200 *Solanum* species is well documented [4–6]. They occur in most plant tissues including the fruit, leaf, bud, flower and stem [5], and also in *in vitro* tissue cultures [7], where they are thought to play a protective role as deterrents of certain insects [2]. The larval inhibitory effect of tomatine has been summarized [2, 8]; the activity of solamargine, solasonine, solamarines and/or their corresponding aglycones was assessed on Colorado potato beetle [9, 10], potato leafhopper [10], spiny bollworm [11], jowar stem borer [12], the bug *Dysdercus similis* [13], termites [14] and the snails *Lymnaea cubensis* and *Biomphalaria glabratus* [15]. The spirosolane aglycones solasodine, tomatidenol and tomatidine have a conventional steroidal frame with a spiro-azaketal functionality present in the side chain (Fig. 1, **1–3**). The isomers solasodine (22*R*, 25*R*) and tomatidenol (22*S*, 25*S*) occur mostly as similarly paired triosides, sharing the carbohydrate moieties chacotriose (solamargine and β-solamarine, respectively) and solatriose (solasonine and α-solamarine, respectively) (Fig. 1, **5–8**). Tomatidine, the saturated derivative of tomatidenol, occurs as a 3β-lycotetraoside (tomatine, Fig. 1, **9**).

The biological activity of the spirosolane glycoalkaloids has been variously attributed to their membrane disrupting properties [16–18], or to an inhibitory effect on insect sterol metabolism ascribed [19, 20] to the presence of the imino group in ring F,

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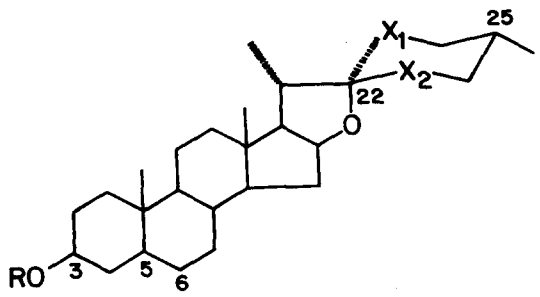

Fig. 1. Structures of spirosolane and spirostan aglycones and glycosides

Compound		A/B rings juncture	R	X_1	X_2
1	Solasodine	Δ^5	H	NH	CH_2
2	Tomatidenol	Δ^5	H	CH_2	NH
3	Tomatidine	5α-H	H	CH_2	NH
4	Diosgenin	Δ^5	H	O	CH_2
5	Solamargine	Δ^5	Chacotriosyl	NH	CH_2
6	Solasonine	Δ^5	Solatriosyl	NH	CH_2
7	β-Solamarine	Δ^5	Chacotriosyl	CH_2	NH
8	α-Solamarine	Δ^5	Solatriosyl	CH_2	NH
9	Tomatine	5α-H	Lycotetraosyl	CH_2	NH

much in the manner of synthetic azasteroids [21] and aziridines [22, 23]. In order to evaluate these potential functions, a study has been undertaken of the effect of spirosolane derivatives on larvae of a major stored-product pest, the red flour beetle, *Tribolium castaneum* Herbst (Coleoptera) and, in parallel, of the oligophagous tobacco hornworm, *Manduca sexta* Johan. (Lepidoptera, Sphingidae). The latter had served as a larval test system for azasteroid studies [21], and thus provides a convenient reference model for comparison with the effect of spirosolane derivatives.

RESULTS AND DISCUSSION

Preliminary experiments conducted with crude, dry extracts from leaves and fruits of various spirosolane derivative-containing *Solanum* plants showed a significant inhibitory effect on larval growth of *T. castaneum* without affecting pupation or emergence (Table 1). The results suggested that the active compounds in the extracts might be solamargine, solasonine and solamarines. This inferrence was substantiated by lack of activity of the fenugreek (*Trigonella foenum graecum*) seed extract which contains glycosides of the non-alkaloidal steroid diosgenin, a ring F-oxygen analogue of solasodine (Fig. 1, 4).

Further experiments with pure samples showed that at dietary concentrations of 1 μmol g^{-1}, solamargine, solasonine and tomatine inhibited larval growth of *T. castaneum* without affecting pupation or emergence (Table 2), as seen previously with the related crude plant extracts (Table 1). Neither the aglycones (solasodine, tomatidenol, tomatidine and diosgenin) nor the carbohydrate moiety components (Fig. 2) (D-glucose, L-rhamnose, D-galactose and D-xylose) of the tested glycosides were found to affect the larval growth

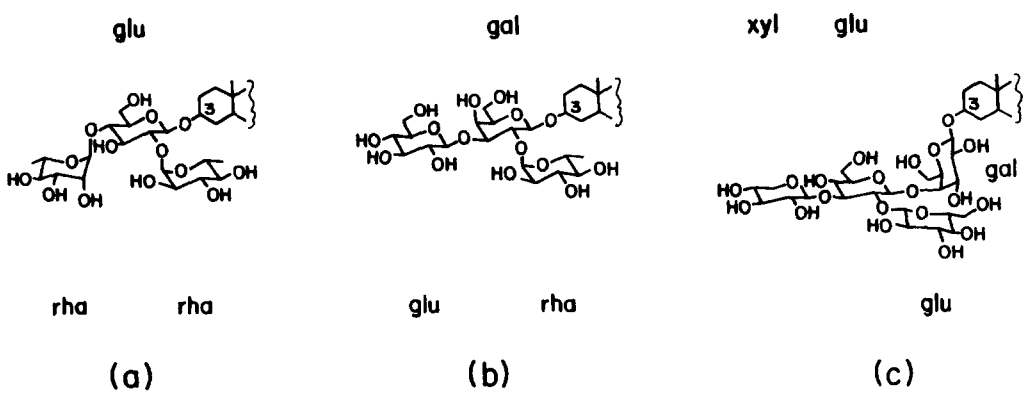

Fig. 2. Carbohydrate moieties attached to the position 3 in the steroidal framework of some spirosolane glycoalkaloids. (a) chacotriose; (b) solatriose; (c) lycotetraose. glu = D-glucose; gal = D-galactose; xyl = D-xylose; rha = L-rhamnose.

Table 1. Effect of crude, dry plant extracts on larval weight, pupation and emergence of *Tribolium castaneum*

Plant source	Plant organ	Dietary concentration (ppm)	Weight	Pupation	Emergence
			(% of control)		
Lycopersicon esculentum Mill.	Leaf	500	72±4**	98	98
Solanum laciniatum Ait.	Leaf	500	74±4**	100	100
Solanum aviculare Forst.	Leaf	500	85±5*	100	100
Solanum khasianum Clarke	Fruit	500	89±3*	100	97
Solanum mammosum L.	Fruit	500	94±2	98	98
Solanum marginatum L.	Fruit	500	97±4	98	98
Solanum dulcamara L.†	Leaf	500	95±2	100	100
Trigonella foenum graecum L.	Seed	500	98±3	100	100

Data are the means and s.e. values of five replicates of 10–12 larvae each. The average larval weight of the control was 2.30±0.10 mg, with 100% pupation and emergence.

* Significantly different from untreated control at $p = 0.05$.

** Significantly different from untreated control at $p = 0.01$.

† Raw glycoalkaloids mixture.

Table 2. Effect of β-sitosterol and of cholesterol on the inhibitory action of solamargine, solasonine and tomatine on larval weight, pupation and emergence of *Tribolium castaneum*

Compound	Dietary concentration (μmol g^{-1} diet)	Dietary concentration (ppm)	Weight	Pupation	Emergence
			(% of control)		
Solamargine	1.0	867	74±2[a]	100	94
Solamargine+sitosterol	1.0+1.0	867+415	108±3[b]	100	97
Solamargine+cholesterol	1.0+1.0	867+387	96±4[b]	100	100
Solasonine	1.0	884	64±4[a]	97	97
Solasonine+sitosterol	1.0+0.1	884+41.5	70±5[a]	96	96
Solasonine+sitosterol	1.0+1.0	884+415	71±5[a]	96	97
Solasonine+cholesterol	1.0+0.1	884+38.7	54±3[a]	93	92
Solasonine+cholesterol	1.0+1.0	884+387	63±4[a]	94	93
Tomatine	1.0	1034	60±5[a]	96	96
Tomatine+sitosterol	1.0+0.1	1034+41.5	69±6[a]	100	100
Tomatine+sitosterol	1.0+1.0	1034+415	102±5[b]	100	100
Tomatine+cholesterol	1.0+0.1	1034+38.7	90±8[b]	100	100
Tomatine+cholesterol	1.0+1.0	1034+387	107±6[b]	100	100
Cholesteryl tomatide	1.0	1034+387	117±4	98	98
Cholesteryl tomatide	2.0	2068+674	48±4**	89	88

Data are the means and s.e. values of ten replicates of 10–12 larvae each. The average larval weight of the control was 1.93±0.06 mg, with 98±1% pupation and 96±3% emergence. Dietary levels of β-sitosterol and cholesterol had no effect on larval growth, pupation or emergence, when given separately.

[a] and [b] represent data which differ significantly ($p = 0.05$) from each other within the same group.

** Significantly different from untreated control at $p = 0.01$.

of *T. castaneum* (Table 3). No selectivity in the inhibitory effect which could, conceivably, be correlated with the configuration at C-22 of the nitrogen atom, was actually discerned.

The inhibitory effect of solamargine and tomatine on larval growth was completely abolished by addition of 1 μmol g^{-1} diet cholesterol or sitosterol (Table 2). At 0.1 μmol g^{-1} diet, cholesterol was more effective than sitosterol at reversing the inhibitory effect of tomatine. Conversely, cholesterol and sitosterol did not affect the inhibitory action of solasonine. Incidentally, *in vitro* studies demonstrated that tomatine binds cholesterol less readily than sitosterol [24], while solasonine displayed a much reduced sterol binding capacity compared with solamargine [17, 25].

Assuming that the biological activity of the spirosolane glycoalkaloids is due to their ability to disrupt sterol-containing membranes, it seemed of inter-

Table 3. Effect of aglycones and carbohydrate moiety components of steroidal glycosides on larval weight, pupation and emergence of *Tribolium castaneum*

Compound	Dietary concentration (μmol g^{-1} diet)	Dietary concentration (ppm)	Weight	Pupation	Emergence
			(% of control)		
Solasodine	1	414	87±6	100	86
Tomatidenol	1	414	98±2	100	100
Tomatidine	1	416	87±6	100	100
Diosgenin	1	415	91±3	100	100
D-Xylose	3	450	103±3	100	100
D-Galactose	3	540	94±4	100	98
L-Rhamnose	3	492	101±3	100	100
D-Glucose	3	540	100±3	100	97

Data are the means and s.e. values of five replicates of 10–12 larvae each. The average larval weight of the control was 2.36±0.05 mg, with 100% pupation and emergence.

est to explore the behavioural response of *T. castaneum* larvae to an already formed glycoalkaloid-sterol complex. One such derivative which could be isolated in crystalline form is cholesteryl tomatide, the equimolar complex of tomatine with cholesterol [26], and although inactive at 1 μmol g^{-1} diet, it displayed significant activity at 2 μmol g^{-1} diet (Table 2). This result could imply that complex formation between glycoalkaloids and membrane sterols, though occurring readily *in vitro*, is not necessarily the only condition for the former's activity *in vivo*. Another determinant might be the aglycone moiety, either by itself or through interaction with the carbohydrate unit. The latter possibility is derived from features such as differential membrane-lytic [17] and antifungal [18] activity of triose glycoalkaloids in both spirosolane and solanidane series. Another view-point is that the aglycone liberated by surface glycosidases is the active moiety [27], and indeed experiments have revealed antifungal [19, 28–30], enzyme inhibitory [20], and membrane disruption [31] activity of aglycones. The last report also implicated a different type of interaction of the active aglycone with membranes, as compared with the corresponding glycosides which are held to act stepwise by insertion of the aglycone part of the glycoalkaloid in the bilayer's membrane-embedded sterol component, followed by complexation of the glycoalkaloid with the sterols present; finally, rearrangement of the membrane induced by the formation of domains enriched in sterol-glycoalkaloid complexes, results in a transient disruption of the bilayer during which membrane leakage occurs [31].* Reduced leakage caused by the aglycone compared with its glycosides has been attributed [31] to a lack of intermolecular carbohydrate–carbohydrate interaction between adjacent molecules [32].

The assumption that the spirosolane aglycones might act like azasteroids, which are highly active synthetic inhibitors of insect sterol metabolism [21], was inferred [19, 20] from the presence in the former of a ring F-imino group, which is reminiscent of the three-membered imino ring found in aziridines such as 24,28-imino-fucosterol [22], and 24(*R*,*S*),25-epiminolanosterol [23]. The synthetic azasteroids and aziridines have been demonstrated to inhibit the enzymatic dealkylation of phytosterols, resulting in disturbance of cholesterol biosynthesis and growth inhibition of insects which lack the ability to synthesize sterols *de novo* and require exogenous sterols of both plant and animal origin [21, 22]. Yet, the spirosolane derivatives are not strictly imines but rather spiro-ketimines (Fig. 1), and some of them possess in addition the 3β-hydroxy-5-ene moiety found in various phytosterols. Their teratogenicity and embryotoxicity were attributed, respectively, to the presence of a nitrogen atom bonded in a position analogous to hormone-binding sites and thus accessible to the α face of the steroid plane [33], and to the basicity of the nitrogen atom thought to be involved in binding to cell membrane receptor sites by charge transfer and/or hydrogen bonding interactions [34]. Likewise, the configuration at C-22 might be expected to influence the course of the reactions at the nitrogen atom, as seen by readier *N*-acylation of 22*S*-derivatives compared with their more hindered 22*R*-counterparts [35]. Furthermore, the spiroaminoacetal group is reportedly prone to cleavage, affording an electrophilic iminium ion believed to be capable of alkylating DNA in a mechanism-based yeast bioassay [36].

However, it appeared of interest to explore in more detail the extent to which the spirosolane derivatives might actually mimic azasteroids, and this was done in a comparative study with *M. sexta*, which has been previously employed in synthetic azasteroid studies [21]. The experiments were run with the test compounds **1**, **3**, **5**, **6** and **9** at 260 ppm and 1040 ppm without the additional sitosterol usually included in

* A similar mechanism has been previously proposed for the interaction of the non-nitrogen steroidal glycoside digitonin with membrane sterols [32].

Table 4. Major sterol profile in *Manduca sexta* larvae reared on diet containing spirosolane alkaloids and glycoalkaloids

Compound	Insect sterols (relative % of total)				
	Cholesterol	Desmosterol	Campesterol	Sigmasterol	Sitosterol
(Control)	77.1	Tr.	5.5	Tr.	17.4
Solasodine	47.4	2.2	16.3	Tr.	34.2
Tomatidine	40.9	3.3	14.5	1.8	39.5
Solamargine	43.6	2.1	15.3	0.8	37.6
Solasonine	52.9	2.6	16.4	Tr.	28.1
Tomatine	41.0	Tr.	19.8	Tr.	39.1

Test compounds fed at 1040 ppm; larvae sacrificed at 15 days; Tr., traces, <0.5%.

the diet, in order to avoid a possible abolition of activity of the type encountered with *T. castaneum* (see above); all the same, the diet still contained minimal amounts of endogenous phytosterols from the wheat germ which is required for satisfactory growth and development. Tests with insects fed sitosterol-supplemented diet were run simultaneously and little difference was seen compared with tests without added sitosterol. The test was begun at 260 ppm, since that is the concentration of sitosterol normally added to all the diets, including the controls. Tomatine was the only one of the five compounds that demonstrated a notable effect, though only at the higher concentration (1040 ppm), by inhibiting larval weight and development (3% weight gain and 33% pupation, respectively, relative to untreated control). In another experiment done solely with tomatine and tomatidine (results not tabulated), no pupae at all were obtained in the tomatine test at 1040 ppm (1 μmol g^{-1} diet), whereas 50–67% of insects on the tomatidine-supplemented diet did pupate and produce adults. Again, supplementing tomatine and tomatidine with sitosterol made little difference to the development of the insects fed these compounds. The overall rate of growth was somewhat slower in the tests where there was no added dietary sitosterol, but there were adequate endogenous phytosterols such as sitosterol and campesterol for the insects to complete normal development to about the same extent in all but the tomatine-fed insects.

Table 4 includes the percentages of the major sterols in *M. sexta* larvae fed the test compounds at 1040 ppm. Compared with controls, there is a considerable reduction in total cholesterol which would normally comprise over 75% of the total sterols when using the diet supplemented with sitosterol [37]. The test compounds may interfere with the uptake of the phytosterols at such a high concentration of the former. However, there is no accumulation of desmosterol (cholesta-5,24-dien-3β-ol) that would occur if the 24-reductase enzyme was inhibited, as seen with synthetic azasteroids or alkyl amines [21]. Also, there would be considerably less cholesterol produced following inhibition of dealkylation with synthetic azasteroid or alkyl amine inhibitors at only 1–5 ppm concentration in the diet [21]. Since the synthetic azasteroids affect sterol metabolism and larval development at much lower concentrations than the spirosolane derivatives, it seems that the inhibitory effect of tomatine on both features is not based on an azasteroid-type mechanism. At any rate, these results contrast with earlier reports which disclosed slight feeding stimulation activity in *M. sexta* larvae by tomatine, both behaviourally (in food choice-tests using filter paper discs laced with 1 mM test solution [38]) and electrophysiologically (at 0.1 mM) [39]; the aglycone tomatidine was not active in either test. An ecologically intriguing induced preference was seen for tomatine, as it slightly stimulated feeding in *M. sexta* larvae reared on tomato, but slightly deterred feeding in larvae reared on Jerusalem cherry, *Solanum pseudocapsicum* L. [38].

Presumably, the inhibitory effect of the tested spirosolane glycoalkaloids on larval growth of both *T. castaneum* and *M. sexta* could be associated with poor utilization of the food, though a decrease in food uptake resulting from antifeedant activity of the compounds, can also be envisaged. A comparison between different chacotriose- and/or solatriose-based glycoalkaloids in their effects on target membranes, highlights the importance of the carbohydrate moiety (but also of the aglycone through its interaction with the latter, the pH response, and the type of membrane sterol) to sterol-mediated membrane disruption [17]. The subtle balance of these factors is apparent in the synergistic effect of the naturally paired, chacotriose/solatriose-based glycoalkaloids on various living and non-living membrane-bound structures [16–18], which might have major allelochemical significance.

EXPERIMENTAL

Tests compounds. Tomatidine, diosgenin and tomatine were of commercial origin (Fluka AG, Buchs, Switzerland). Solasodine, solamargine and solasonine were prepd from berries of *Solanum khasianum* according to an original procedure [3], and identified by reference to authentic samples. Tomatidenol was prepd according to the same procedure [3] from a mixt. of glycosides obtained from leaves of *Solanum dulcamara*, and identified by reference to an authentic

sample kindly provided by Dr Irmgard Merfort, University of Düsseldorf, Germany. Cholesteryl tomatide was prepd according to a reported procedure [26]. Crude, dry plant extracts were obtained following extraction with EtOH of dried, powdered fruits and/or leaves of *Lycopersicon esculentum*, *Solanum laciniatum*, *Solanum aviculare*, *S. khasianum*, *Solanum mammosum*, *Solanum marginatum* and *T. foenum graecum* plants grown in experimental field at The Volcani Center, A.R.O., Bet Dagan, Israel, and harvested at about eight months after sowing [40]. A sample of *S. dulcamara* glycosides was obtained through the courtesy of Prof. J. F. Verbist, Faculty of Pharmacy, University of Nantes, France.

Insect rearing and bioassay. (a) *T. castaneum.* The rearing procedure, using wheat flour containing 5% dried yeast (Matan Ltd., Hadera, Israel) as basic diet, was described previously [41]. For bioassay, 10 g diet was mixed with 10 ml of a soln of the test extract and/or compound in EtOH (or CH_2Cl_2 for aglycones) at appropriate concns, and/or with 10 ml of the corresponding solvent as control. Following solvent evapn and thorough mixing, the diet was distributed in 2 g portions in test vials. 10–12 larvae 0–3 hr after hatching were placed in each test vial, and kept at 28°. The live larvae were weighed at 16 days as a group, and the average wt ± s.e. of the means was calcd from five replicates; they were then returned to the diet for determination of pupation and adult emergence. (b) *M. sexta.* The rearing procedure using a mixt. of extracted casein, brewer's yeast, wheat germ and sitosterol (0.026% wet wt, or 0.2% dry wt) as basic diet, was described previously [21] and employed six larvae per test. The test compounds were 'coated' on the dry components of the diet at 260 and 1040 ppm concns. Larvae were weighed on day 15 and examined for inhibition of larval development, abnormal prepupal–pupal forms, and sterol analysis to determine possible effects of dealkylation. A similar diet, but without added sitosterol, was used in parallel in order to avoid possible interaction with certain test compounds; however, there was still a need to use in the diet some wheat germ which is essential for satisfactory growth and development, and usually contains approximately 7–8 mg of endogenous phytosterols, mostly sitosterol and campesterol (*ca* 3 : 1), per 100 ml of diet. Without being sterol-free, this diet does limit endogenous sterol to the minimum extent possible.

Statistical analysis. The results were subjected to one-way analysis of variance (ANOVA), and means were sepd by Scheffé's multiple range test ($P = 0.05$) [42]. Angular transformation for percentage pupation and emergence was done before statistical analysis.

Insect sterols analysis. M. sexta sterols were extracted from insects reared on the test diets and sacrificed at 15 days, and then analysed by GC-MS, as previously reported [43].

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